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Comprehensive pharmacokinetics of a humanized antibody and analysis of residual anti-idiotypic responses

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SUMMARY

A murine antibody to human tumour necrosis factor-α (TNF-α) (CB0010) was complementarity-determining region (CDR)-grafted using human IgG4 heavy and κ light chain constant regions. In cynomolgus monkeys, the grafted antibody (CDP571) was eliminated with a half-life of 40–90 hr, two to three times longer than CB0010, and immunogenicity was reduced by > 90%. Responses to the constant regions were almost entirely eliminated and responses to the CDR loops (anti-idiotype) were lowered. CDP571 was given to 24 human volunteers in doses from 0·1 to 10·0 mg/kg. It was well tolerated, with a half-life of approximately 13 days. Anti-CDP571 antibodies were low or undetectable at higher doses. At lower doses, anti-CDP571 peaked at 2 weeks and then declined. The response was primarily IgM (in contrast to the cynomolgus monkey, where by 5 weeks IgG predominated) and was against a conformational epitope comprising heavy and light chain CDR loops. No antibodies were detected against the γ_4/κ domains or frameworks. The response had little or no effect on CDP571 binding to TNF-α or on plasma clearance.

INTRODUCTION

Therapeutic effects of murine monoclonal antibodies have been demonstrated in several diseases, using antibodies to cancer antigens,[1] cell-surface molecules,[2] cytokines[3] and infectious agents.[4] However, their potential in chronic diseases is limited by a short half-life (1–2 days), poor effector function recognition and the development of human anti-mouse antibodies[5,6] that restrict repeated use.

A number of techniques has been developed to reduce the rodent component of antibodies.[7–9] Chimerization of antibody 17–1A (against a glycoprotein on gastrointestinal cancer cells) resulted in a sixfold increase in circulation time and a considerable reduction in immunogenicity.[10] However, the variable region of the murine antibody retained the potential to be immunogenic.[11] Rodent-derived sequences can be reduced further by integrating the complementarity-determining regions (CDR), forming the antigen-binding site, into human V-region frameworks.[8,12,13] The initial loss in affinity could generally be restored by replacing specific amino acid residues adjacent to the CDR.[14] Comparison of a murine and CDR-grafted anti-Tac antibody demonstrated a prolonged half-life and reduced immunogenicity in monkeys.[12] We have applied these techniques to an antibody to human tumour necrosis factor-α (TNF-α), for which there are a number of potential disease indications in humans.[15–18] CDR from the murine antibody (CB0010) were expressed in a human framework with human γ4 heavy and κ light chain constant regions (CDP571). Comparison of these molecules in monkeys and administration of the grafted molecule to healthy human volunteers has confirmed a considerable increase in half-life and reduction in immunogenicity, thus increasing the potential for repeated therapy.

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Abbreviations: CDR, complementarity-determining region; HRP, horseradish peroxidase; H, heavy; L, light; rhTNF-α, recombinant human tumour necrosis factor-α; TMB, tetramethyl benzidine.

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MATERIALS AND METHODS

Generation of CDP571 antibody

CB0010 was raised in BALB/c mice against recombinant human TNF-α (rhTNF-α) produced in *Escherichia coli*. The genes for the heavy (H) and light (L) chains were isolated from a cDNA library and their sequences determined. The CDR were defined according to Kabat *et al.*[19] A computer graphic analysis of models constructed for the murine and human variable regions was used to identify framework residues capable of contacting antigen and maintaining the conformational integrity of the CDR.[20] The CDR, together with these additional residues, were transferred into the framework of the human antibody Eu (Fig. 1) with human γ4 H chain constant regions and a κ L chain. Genes encoding the humanized variable domains were constructed using a series of overlapping oligonucleotides, which were assembled using polymerase chain reaction.[21] The engineered antibody retained the full binding activity for TNF-α, as assessed by competition enzyme-linked immunosorbent assay (ELISA) and neutralization in a cytotoxicity assay (murine fibroblast cell line, L929).

Heavy chain sequences

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Eu       QVQLVQSGAE VKKPGSSVKV SCKASGGTFSRSAII WVRQA PGQGLEWMGG
CB0010   EVLLQQSGPE LVKPGASVKI PCKASGYTFTDYNVD WVKQS HGKSLQWIGN
CDP571   QVQLVQSGAE VVKPGSSVKV SCKASGYTFTDYNVD WVKQA PGQGLQWIGN

Eu       IVPMFGPPNYAQKFQG  RVTITADESTNTAYMELSSLRSED TAFYFCAGGY
CB0010   INPNNGGTIYNQKFKG  KGTLTVDKSSSTAYMELRSLTSED TAVYYCARSA
CDP571   INPNNGGTIYNQKFKG  KGTLTVDKSTSTAYMELSSLTSED TAVYYCARSA

Eu       GIYSPE            EYNGGLVTVSS
CB0010   FYNNYEYFDV        WGAGTTVTVSS
CDP571   FYNNYEYFDV        WGQGTTVTVSS
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Light chain sequences

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Eu       DIQMTQSPST LSASVGDRVT ITCRASQSI. ....NTWLA WYQQKPGKAPK
CB0010   DIMMSQSPSS LAVSVGEKVT MSCKSSQSLLYSNNQKNYLA WYQQKPGQSPK
CDP571   DIMMTQSPST LSASVGDRVT ITCKSSQSLLYSNNQKNYLA WYQQKPGQAPK

Eu       LLMYKASSLES GVPSRFIGS GSGTEFTLTI SSLQPDDFAT YYCQQYNSDS
CB0010   LLISWASTRES GVPDRFTGS GSGTDFTLTI SSVKAEDLAV YYCQQYYDYP
CDP571   LLISWASTRES GVPSRFIGS GSGTEFTLTI SSLQPDDVAT YYCQQYYDYP

Eu       KMFGQGTKVE VKG
CB0010   WTFGGGSKLE IKR
CDP571   WTFGQGTKVE IKR
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Figure 1. Aligned amino-acid sequences of heavy and light chain variable regions. Eu, human acceptor antibody; CB0010, murine parent antibody; CDP571, grafted antibody. The CDR regions are single underlined, with the framework positions double underlined. The residues comprising the CDR are based on Kabat *et al.*[19]

An expression vector was constructed, containing H and L chain coding sequences under the control of separate cytomegalovirus-major immediate early (CMV-MIE) promotor-enhancers and a glutamine synthetase selectable marker. The expression plasmid was essentially pST-6,[22] in which the V_H and V_L coding regions had been substituted with those of the anti-TNF antibody. In addition, 3'-flanking DNA from the mouse immunoglobulin μ gene was inserted between the H and L chain transcription units to serve as a transcription termination signal. The expression plasmid was transfected into NS0 myeloma cells and glutamine-independent transfectants isolated as described previously.[22]

Reagents

For anti-CDP571 and anti-CB0010 standards, rabbits were injected subcutaneously (s.c.) at 4 monthly intervals with 100 μg CDP571 or CB0010 in incomplete Freund's adjuvant. Serum was affinity purified on Sepharose (Pharmacia, Sweden) coupled with the homologous antibody, according to the manufacturer's protocol. For anti-CDR standards, anti-CDP571 serum was purified on CB0010–Sepharose. Antibodies were then biotinylated (Biotin-X-NHS; Calbiochem, La Jolla, CA), or directly conjugated to horseradish peroxidase (HRP) using iminothiolane and HRP-maleimide. Tetramethyl benzidine (TMB) substrate was stabilized using β-cyclodextrin.

Molecules used for epitope mapping were: CDP571, CB0010, CB0006 (murine IgG1 anti-TNF, unrelated idiotype), cB72.3 (chimeric antibody, unrelated idiotype, human γ4 constant regions), gB72.3 (CDR-grafted, Eu framework and γ4 constant regions) and gH + gL (immunoglobulin from cells transfected with mixed H and L chains from CDP571 and gB72.3).

Cynomolgus monkey study

Pairs of male cynomolgus monkeys (*Macaca fascicularis*) received either CDP571 at 0·1, 1·0 and 10·0 mg/kg or CB0010 at 0·1 mg/kg. Plasma samples (EDTA) were collected relative to antibody infusion at days −4 and 0 (pre-dose), 1, 3, 5, 10, 20

and 40 min, 1, 3, 6, 12 and 24 hr, daily until day 7, and thereafter on days 10, 14, 21, 28 and 34. Samples were stored at −70° until assay.

Human volunteer studies

Six groups of six healthy males were infused intravenously (i.v.) over 30 or 60 min with ascending doses of CDP571 ($n = 4$) at 0·1, 0·3, 1·0, 2·0, 5·0 and 10·0 mg/kg or blinded placebo ($n = 2$). Plasma samples were collected relative to the end of antibody infusion at pre-dose, 0 min (end infusion), 5, 10, 20, 30, 60 min, 3, 6, 12, 18, 24 hr, 2, 3, 4, 5, 6, 7, 10, 14, 21, 28 and 35 days, and plus 3 months (exluding the 0·1 mg/kg group).

Pharmacokinetic ELISA

Microtitre plates (Nunc Maxisorb Immunomodule, Kamstrup, Denmark) were coated with rhTNF-α (0·5 μg/ml; Bissendorf, Hannover, Germany) and blocked with bovine serum albumin (BSA). Samples were serially diluted in phosphate-buffered saline (PBS)/1% BSA and tested in duplicate. Bound CDP571 was revealed with a murine monoclonal antibody to human IgG4 (Serotec, Kiddlington, UK). CB0010 and CDP571 assays were completed with HRP-conjugated goat anti-mouse IgG (Fc-specific; Jackson Laboratories, Westgrove, PA) and TMB substrate. The limit of detection for this assay was 5 ng/ml.

Statistics

Model-independent pharmacokinetic parameters of CDP571 were calculated using a SAS program (SAS Institute Inc., Cary, NC). The area under the plasma concentration–time curve (AUC) was calculated using the trapezoidal rule. AUC ($t-\infty$) was determined by extrapolating to infinity from C_t assuming exponential decay using the equation AUC ($t-\infty$) = C_t/λ_z, where C_t = concentration at last measured sample and λ_z = elimination rate constant. AUC ($0-\infty$) was derived from AUC ($0-t$) + AUC ($t-\infty$). Elimination half-life was calculated as $\ln(2)/\lambda_z$. λ_z was calculated by linear regression analysis of the natural logarithm of CDP571 concentration against time since the start of antibody infusion.

Detection and characterization of antibodies to CDP571

A double-antigen sandwich ELISA was used. Samples were diluted 1/10 and added to microtitre plates coated with CDP571 (0·5 μg/ml). Bound antibodies were reacted with the CDP571–HRP (125 ng/ml) followed by TMB substrate. Results are expressed as U/ml, where 1 U is equivalent to 1 μg/ml rabbit standard. This assay will detect 50 ng/ml polyclonal goat anti-human IgG (I2136; Sigma, Poole, UK). For anti-idiotype assays, CB0010–HRP replaced CDP571–HRP and rabbit anti-CDR standards were used. Anti-CB0010 in monkeys was measured using CB0010 on both sides of the assay. For epitope mapping, CDP571-biotin followed by HRP–streptavidin replaced CDP571–HRP. Competing molecules (100-fold excess) were added with the CDP571–biotin. Results are expressed as percentage of the signal with CDP571–biotin alone.

HPLC analysis

One-hundred microlitres of filtered plasma was applied to a size exclusion column (Zorbax GF250XL, range 4000–400 000 MW; Anachem, Luton, UK), run at a flow-rate of 1·0 ml/min with 0·2 M Na_2PO_4, pH 7·0, on a 1090 M liquid chromatograph with a high-performance liquid chromatography (HPLC) Chemstation (HP79994A; Hewlett-Packard Ltd, Waldbrown, Germany). The column was calibrated for IgM with human IgM myeloma (Jackson Laboratories, Westgrove, PA), and for IgG with CDP571. Ten 0·5-ml fractions were collected starting 6 min into each run.

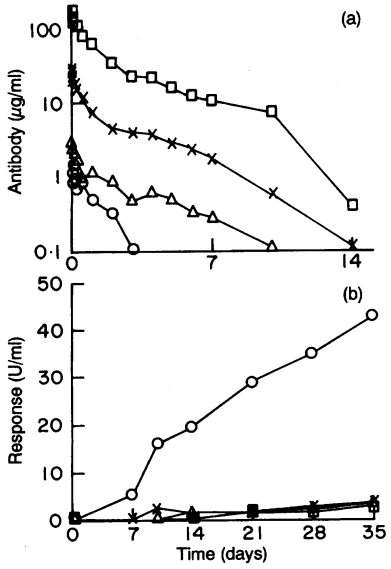

Figure 2. Pharmacokinetics and immunogenicity of CB0010 and CDP571 in cynomolgus monkeys. (a) Elimination profiles for CB0010 and CDP571. (b) Immune responses to CB0010 and CDP571. Animals were given single i.v. infusions as follows ($n = 2$ per group): (○) CB0010 0·1 mg/kg; (△) CDP571 0·1 mg/kg; (×) CDP571 1 mg/kg; (□) CDP571 10 mg/kg.

RESULTS

Pharmacokinetics in the cynomolgus monkey

CDP571 and CB0010 elimination profiles are illustrated in Fig. 2a. Peak drug levels were achieved within 5 min; the $\beta t_{1/2}$

Table 1. Summary pharmacokinetics of CB0010 and CDP571

Dose (mg/kg)	Species	Antibody	$\beta t_{1/2}$ Hours	$\beta t_{1/2}$ Days	C_{max}* (μg/ml)	AUC $0-\infty$ (μg/hr/ml)
0·1	Monkey	CB0010	27·4	1·1	3·4	33
0·1	Monkey	CDP571	65·9	2·7	3·4	93
1	Monkey	CDP571	59·3	2·5	18·7	696
10	Monkey	CDP571	82·4	3·4	182·6	6547
0·1	Human	CDP571	121	5·0	2·5	236
0·3	Human	CDP571	143	6·0	9·1	1058
1	Human	CDP571	165	6·9	25·5	3968
2	Human	CDP571	342	14·3	52·7	11 184
5	Human	CDP571	266	11·1	166·8	26 539
10	Human	CDP571	312	13·0	272·3	49 729

* C_{max}, maximal plasma concentration of CDP571.

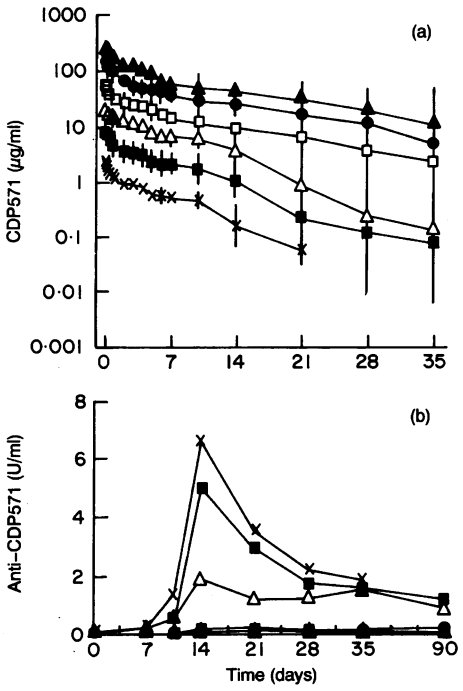

Figure 3. Pharmacokinetics and immune responses to CDP571 in human volunteers. (a) Elimination profiles. (b) Anti-CDP571 responses. CDP571 was administered as a single i.v. infusion at the following doses (n = 4 per group): (×) 0·1 mg/kg; (■) 0·3 mg/kg; (△) 1 mg/kg; (□) 2 mg/kg; (●) 5 mg/kg; (▲) 10 mg/kg. Results are expressed as geometric means (±95% CI).

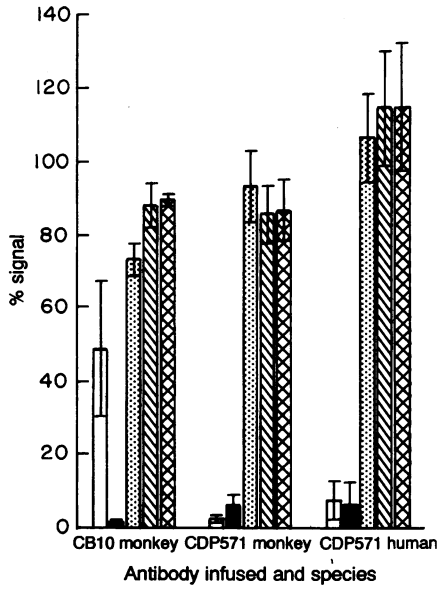

Figure 4. Epitope mapping of immune responses to CB0010 and CDP571. Samples were assayed in the presence of 100-fold excess of the following competitor molecules: (□) CDP571; (■) CB0010; (▨) CB0006; (▧) cB72.3; (▩) gB72.3.

for CB0010 was 27 hr, and for CDP571 40–90 hr, i.e. two to three times longer (Table 1). At the highest dose (10 mg/kg) there was some indication of a more rapid clearance from day 10. AUC calculations, which give an indication of the bioavailable dose, showed proportionality for the three CDP571 groups.

Immunogenicity in the cynomolgus monkey

Anti-CB0010 antibodies were detected from 6 days, increasing over 34 days to 42·5 U/ml (Fig. 2b). Animals receiving CDP571 showed a reduced response, with maximal levels at day 34 of 3·5, 3·7 and 2·8 U/ml for 0·1, 1 and 10 mg/kg, respectively (92% reduction at 0·1 mg/kg). Anti-idiotype antibodies were similar in profile and magnitude to those against the whole molecules, with a reduced response when the idiotype was presented in the human framework (data not shown).

Pharmacokinetics in human volunteers

CDP571 peaked within 10 min of the end of infusion (Fig. 3a), and C_{max} and AUC values indicated dose proportionality across all groups (Table 1). For the three lowest doses (0·1, 0·3 and 1 mg/kg), the elimination rate was not constant in 5/12 subjects, partly due to the limit of assay sensitivity and partly because the antibody cleared more rapidly between 14 and 21 days. Using all time points to calculate the half-life gave an average $\beta t_{1/2}$ of 6 days. For the higher doses (2, 5 and 10 mg/kg), the CDP571 elimination rate was constant, and mean $\beta t_{1/2}$ was 14·3, 11·1 and 13 days, respectively (range 5·6–22·4 days, 134–537 hr). CDP571 remained detectable in the plasma of all 12 volunteers receiving higher doses at 35 days and for 7/12 at 3 months.

Immunogenicity in human volunteers

Anti-CDP571 antibodies were detectable in all patients receiving 0·1 mg/kg CDP571, but decreased with increasing dose. Geometric means for each dose group are illustrated in Fig. 3b. Responses peaked at 14 days and then declined. Anti-idiotype responses showed a similar profile and magnitude (data not shown).

Epitope mapping of immune responses

In monkeys receiving CB0010, responses to murine constant regions were demonstrated by only partial blocking by CDP571 (50%), and a 30% reduction in signal with an unrelated murine IgG1 antibody, CB0006 (Fig. 4). The remaining response was directed against the variable regions. In monkeys receiving CDP571, 90% inhibition by CB0010 indicated that virtually all the response was against the idiotype. Partial blocking (10%) by the γ4 B72.3 grafts could suggest a weak response to human constant regions.

In humans, CB0010 and CDP571 competed equally for the binding of anti-CDP571 antibodies and no competition was seen using cB72.3 or gB72.3. These results confirm that the response was directed entirely against the idiotype. In addition, no competition was seen with CB0006, an anti-TNF antibody which binds an overlapping but not identical epitope on the TNF molecule, indicating that the anti-idiotypic response was specific for the CDP571 idiotype.

To determine whether the response was against the H or L

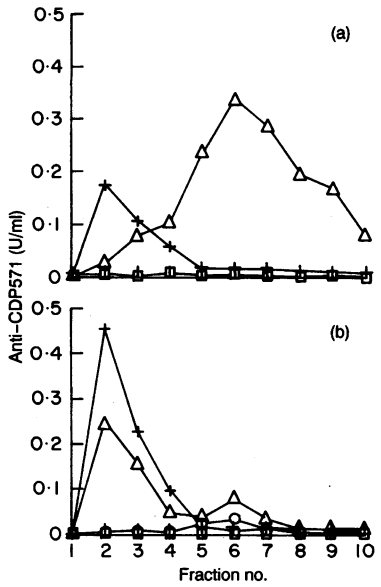

Figure 5. Anti-CDP571 responses in HPLC fractions from sera of a (a) cynomolgus monkey and (b) human volunteer, receiving 1 mg/kg CDP571. Samples were collected at the following times relative to antibody infusion: (□) pre-; (+) 2 weeks post-; (△) 5 weeks post-; (○) 3 months post-.

chain, CDP571 and gB72.3 H and L chains were co-expressed in all four pair-wise combinations. Quaternary assembly of the molecules was confirmed by a sandwich ELISA using anti-Fc and anti-κ antibodies. No inhibition of signal was seen with either B72.3 H/L chain mixtures or CDP571 H with B72.3 L chains, and only 10–20% inhibition was seen with CDP571 L/B72.3 H chains. The CDP571 H/L chain mixture gave 100% inhibition. This indicates that the immune response was directed against an epitope formed by the association of both H and L chain CDR loops of CDP571.

Isotyping of the immune responses

HPLC retention times for IgM were 6·7 min (fraction 2, void volume) and for IgG 8·7 ± 1 min (fractions 5–7). Free CDP571, present in 14- and 35-day samples from volunteers receiving higher doses, eluted in fraction 6 and therefore may have caused an underestimate of the IgG anti-CDP571. Also, any IgG–CDP571 complexes present that did not dissociate during HPLC eluted in fraction 5. This was demonstrated in only one volunteer (5 mg/kg).

Pretreatment, 14- and 35-day and 3-month samples from at least two responding volunteers from each dose group, and samples from four monkeys, were analysed by this method. Typical profiles are illustrated in Fig. 5a, b. In all monkey samples tested, the response switched from predominantly IgM at day 14, to IgG at 35 days post-infusion. By contrast, in humans, at day 35 post-infusion the majority of the response was still detected in the IgM fractions, and this was always lower than the level seen at day 14. Only 5/13 samples tested at day 35 had anti-CDP571 activity in the IgG fractions, and only in one volunteer was the signal higher for the IgG fractions than for IgM. At 3 months, 6/12 volunteers still showing a detectable response gave a weak IgG signal and no IgM signal. However, the relative signal generated by IgM and IgG in the double-antigen sandwich assay is unknown and it is possible that the multimeric nature of IgM amplifies the signal relative to IgG.

DISCUSSION

The kinetics of a CDR-grafted anti-TNF-α molecule, CDP571, were studied in cynomolgus monkeys and humans. In monkeys, where direct comparisons were made with the murine parent antibody (CB0010), humanization resulted in a prolonged half-life and a substantial reduction in immunogenicity, both of the constant regions and of the CDR loops. This confirms the benefits of humanization as shown previously with an anti-Tac antibody, where a similar increase in half-life was obtained in monkeys, with residual immunogenicity confined predominantly to the idiotype.[12]

In human volunteers, CDP571 at doses of 2 mg/kg or greater had a $\beta t_{1/2}$ of 12–14 days, approaching the 21-day half-life for human IgG4[23] and being longer than half-lives quoted for chimeric antibodies (4–9 days)[10,24] and other CDR-grafted antibodies.[25,26] A slightly shorter half-life at doses below 1 mg/kg (5–7 days) resulted from an increased clearance rate between 14 and 21 days, which coincided with the detection of a weak, transient immune response in these subjects. The response was lower than that seen in monkeys and of shorter duration, peaking at 14 days and declining thereafter. We have attempted to compare the magnitude of these responses with those reported in studies of other humanized antibodies[25] by cross-calibration using the same polyclonal anti-IgG standard. However, the detection limit for our assay was approximately 40-fold lower than for the anti-Campath-1H assay, making comparative quantification difficult.

The anti-CDP571 response is specific for a conformational epitope formed by the association of heavy and light chain CDR loops (anti-idiotype), with no response detectable to constant or framework regions. The confinement of the response to the idiotype confirms that constant and framework regions can be rendered immunosilent by CDR grafting. Expression of human antibody genes in the xenogeneic host, NS0, could lead to an abnormal pattern of glycosylation, which in turn might create immunogenic epitopes. However, a similar pattern of glycosylation will also be present on the murine antibody CB0006, yet there is no evidence of any response directed against the carbohydrate moiety on this molecule (Fig. 4). Furthermore, the reduction in anti-idiotype response to CDP571 in the monkeys, compared to CB0010, shows that the intrinsic immunogenicity of the CDR can be significantly modulated by their protein context.

In contrast to the monkeys, where there is a strong switch to the IgG class by 35 days, responses in humans were confined primarily to the IgM serum fraction. The observation of only low IgM anti-idiotypic responses in humans is relevant to the potential for repeated therapy. The weak IgG response is consistent with tolerance of B cells as well as T cells seen with high doses of T-dependent antigens, and this should reduce the magnitude of any secondary response. Two important aspects of the response are the effect that the anti-CDP571 antibodies has on the binding of CDP571 to TNF-α, and on clearance of CDP571 from the circulation. IgM antibodies are generally of

low affinity, and the fact that circulating CDP571 could be detected during and beyond the peak anti-CDP571 response, by means of an assay which relied upon its binding to TNF-α, provides strong evidence that these antibodies neither neutralize the circulating anti-TNF activity, nor cause rapid clearance of CDP571. The development of specific anti-idiotypic antibodies was not associated with any adverse events in the volunteers, and data from other studies suggest that they will not result in adverse reactions on subsequent administration of antibody.[25]

The decreasing antibody response seen with increasing doses of CDP571 is also relevant to prolonged therapy. The observation of tolerance to T-dependent antigens at higher doses of antigen is not new[27] and previous investigators using murine[28] and CDR-grafted[12] monoclonal antibodies have made similar observations. The implications for obtaining optimal therapeutic regimes in humans are very important and are now being investigated further.

Although more than 70 humanized antibodies are now in various stages of research, preclinical or clinical development,[29] information regarding the pharmacokinetics and immunogenicity of these molecules in humans is still sparse. Direct comparisons between murine and grafted antibodies is difficult to obtain in humans as few reagents have been taken into the clinic in both forms. The additional benefit of CDR grafting over chimerization is similarly impossible to estimate directly. Chimeric antibodies have themselves been shown to vary considerably in their immunogenicity[10,12] and the potential to generate antibodies to human framework residues has been demonstrated in mice administered mouse/human chimeric molecules.[11] The development of more rapid CDR-grafting techniques has reduced the time saved by chimerization to a few weeks, so it is now difficult to justify the potential risk of increased immunogenicity. The full benefit of humanization may also be masked by the fact that the antibodies are either being used in immunocompromised patients[13] or they are themselves immunosuppressive.[25] Although anti-TNF antibodies have been shown to prolong cardiac allografts in rats,[30] they are not profoundly immunosuppressive[3] and these results are therefore likely to be relevant to other non-immunosuppressive antibodies, particularly those directed against cytokines and cell-surface molecules. To date, no studies have been published where repeated doses of a CDR-grafted antibody have been given to an immunocompetent patient without the development of some level of immune response. We believe the substantially reduced immunogenicity and longer half-life of a CDR-grafted antibody, demonstrated in these studies, are sufficient to enable prolonged or repeated antibody therapy in chronic diseases.

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